# Investigating environmental sinks of macrolide antibiotics with analytical chemistry

TL Jones-Lepp

United States Environmental Protection Agency

NERL-Environmental Sciences Division

Environmental Chemistry Branch

# Introduction

- Macrolide antibiotics

  - Ranking by prescriptions dispensed (US) -

| | | 2005 | 2004 | 2003 | 2002 |
|---|---|---|---|---|---|
| ▪ Azithromycin | - | 7th | 8th | 5th | 6th |
| ▪ Roxithromycin | - | (widely prescribed Europe & Latin America) | | | |
| ▪ Clarithromycin | - | 206th | | | |

- Possible environmental sinks
  - Wastewater effluents
  - Biosolids
  - Sediments
  - Plants

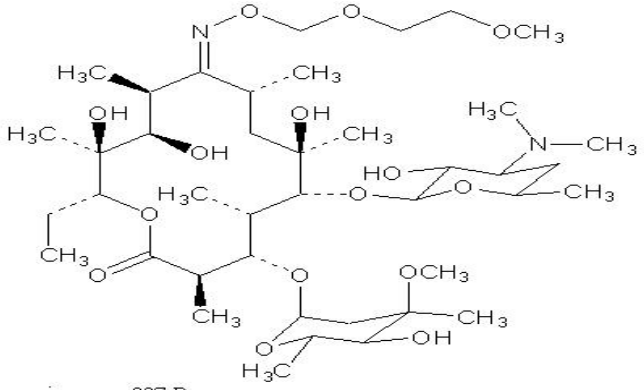
# Why do we care?

- Increasing occurrence of antibiotic-resistant bacteria
  - Miyabara, M. Imoto, S. Arai, J. Suzuki, S. Suzuki, *Environ. Sci.,* 1995, **8**, 171.
  - T. Schwartz, W. Kohnen, B. Jansen, U. Obst, FEMS Microbiology Ecology 2003**, 43**, 325.

- Possible adverse effects due to constant exposure of aquatic organisms to antibiotics

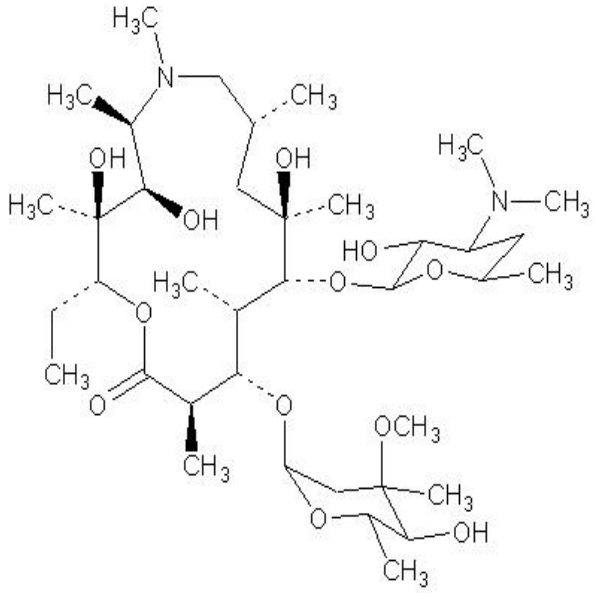
# Chemical structures

Roxithromycin mw = 837.04 Da



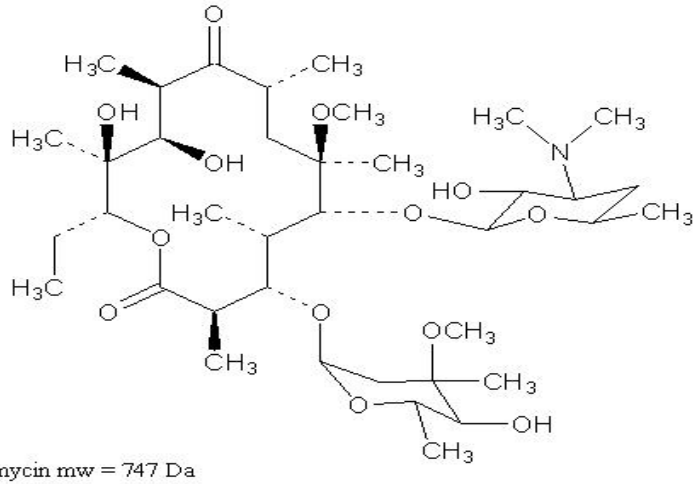
Roxithromycin mw = 837 Da

Azithromycin mw = 748.98 Da



Azithromycin mw = 748 Da

Clarithromycin mw = 747.95 Da



Clarithromycin mw = 747 Da

# Experimental

## Extraction of Waters

### Solid phase extraction

OASIS HLB cartridges, 6-mL capacity, 0.2 g, 30-µm, obtained from Waters Corporation (Milford, MA, USA)

- pH adjust sample to 5.0 to 5.5 pH with 12M HCl
- Wash cartridge – 1 mL/min flow rate
  5-mL methanol --> 5-mL methanol/1% acetic acid --> 5-mL DI water
- Load sample – 5 to 6-mL/min flow rate
- Extract cartridge w/ 3 x 10-mLs methanol/1% acetic acid – 1mL/min flow rate
- Collect eluents in TurboVap tubes place in TurboVap at 23$^{o}$C, 4 psi N2 flow rate, blowdown to 0.5 mL.

# Experimental

## Extraction of Solids

### Pressurized liquid extraction (solids)

•Accelerated Solvent Extraction (ASE) system (Model ASE200, Dionex Corporation, Sunnyvale CA), 33-mL cell size

- •Conditions: 99% methanol/1%acetic acid as the extracting solvent; 2-cycles; 2800psi; 50$^{o}$C
- •Extracts are placed in TurboVap tubes and evaporated under nitrogen (23$^{o}$C, 4psi, Zymark TurboVap) to 0.5 mLs
- •Extracts removed when at 5.0 mL, and washed with 1-mL of hexane x 3 – removing the hexane layer each time – this removes most of the co-extracted lipid material
- •The extract is then placed back into the TurboVap and evaporation is continued to 0.5 mL.

# Experimental

## Liquid chromatography

Column: μ-LC – Restek Allure $C_{18}$, 5-μm particle size, 150 x 3.2-mm
or
Agilent Zorbax RX-$C_{18}$, 3.5 μm particle size, 100 x 2.1 mm

Flow rate = 0.10 mL/min with a 40:60 split after the column,
such that 40% of the flow (40 μL/min) goes to the ES-ITMS

**Gradient elution conditions**

A=99% water/0.1% formic acid
B=82% methanol/18%acetonitrile/0.1% formic acid

| **time min** | **A** | **B** |
|---|---|---|
| 0-2 | 90 | 10 |
| 2-10 | 10 | 90 |
| 10-20 | 10 | 90 |
| 20-28 | 90 | 10 |

5-min equilibrium between analyses

# Experimental

## Detection

Electrospray ionization-ion trap mass spectrometry (ESI-ITMS)

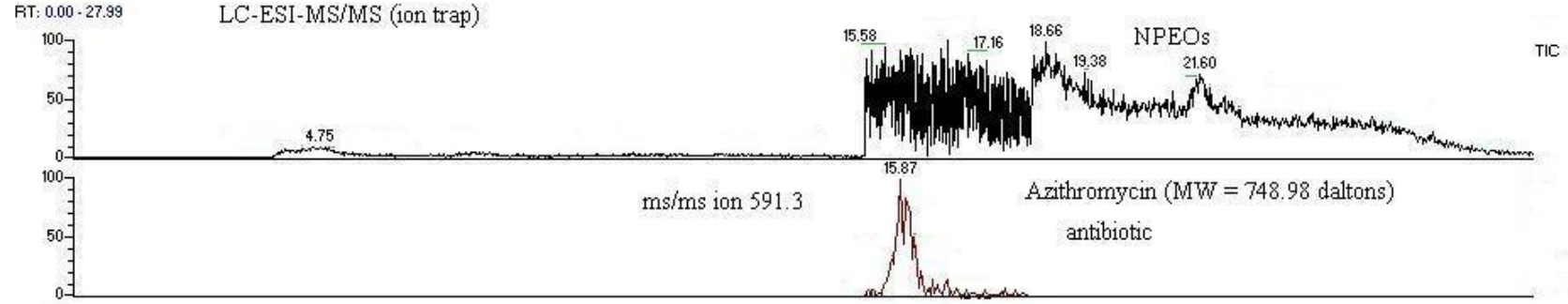
- ThermoFinnigan Classic LCQ
  - Spray voltage 4.8 kV
  - Capillary tube temp 215°C
  - Optimized signal is based on tuning maximum signal from Azithromycin $(M+H)^+$ ion = 749.4

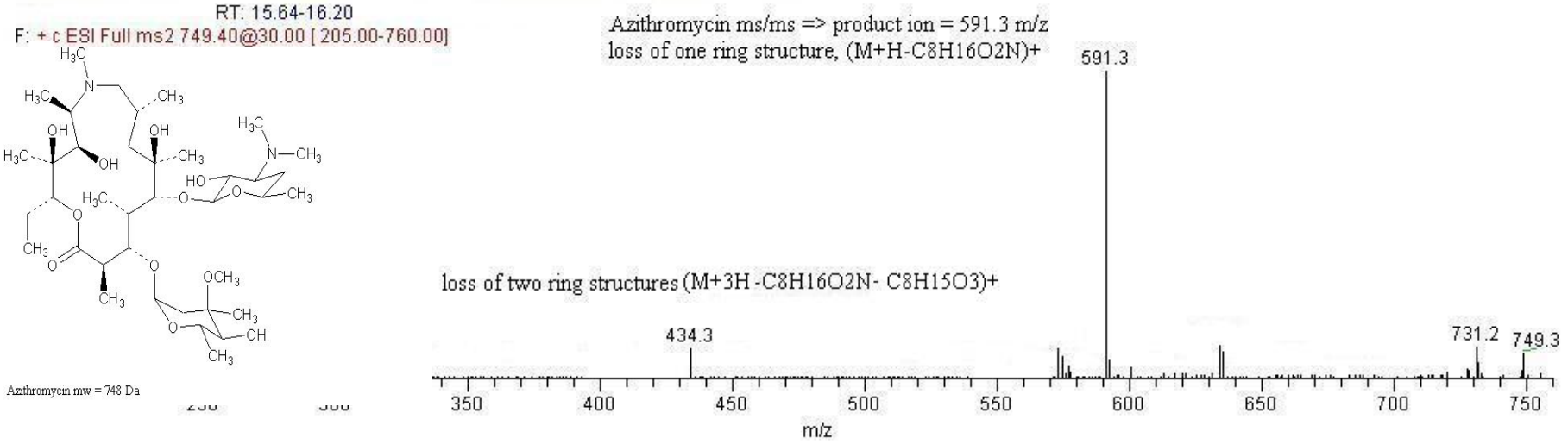
***Macrolide MS/MS product ions for identifying and quantifying***

| Analyte | Precursor ion | Product ion(s) with % relative abundance > 5%; [Collision Energy %] |
|---|---|---|
| Azithromycin | 749.3 $(M+H)^+$ | 591.3 $(M+H-C8H16O2N)^+$ [30]<br>573.3 $(M+H-C8H16O2N-H2O)^+$<br>434.3 $[M+3H-C8H16O2N-C8H15O3]^+$ |
| Clarithromycin | 748.3 $(M+H)^+$ | 590.1 $(M+H-C8H16O2N)^+$ [30]<br>558.2 $(M+H-C8H16O2N-CH3OH)^+$ |
| Roxithromycin | 859.4 $(M+Na-H)^+$ | 755.4 $(M+Na-C4H9O3)^+$ [30]<br>701.4 $[M+Na-C8H15O3]^+$<br>597.4 $(M+Na+H-C4H9O3-C8H16O2N)^+$ |

# Example mass chromatogram and ms/ms spectra

(M+H-C8H16O2N Target analysis of pressurized solvent extraction of a Class A biosolid



# Extraction Recoveries SPE

Water spikes 727ng/L, 500 mL sample

| Sample | Drying time | HCL added | final pH | % avg recoveries Azithromycin | Roxithromycin | Clarithromycin |
|---|---|---|---|---|---|---|
| DI Water | 2 | 50 | 4.5 | 15 | 23 | na |
| DI Water | 20 | 500 | 2.5-3 | 53 | 50 | 41 |
| DI Water | 20 | 100 | 5.0 – 5.5 | 63 | 71 | 74 |
| DI Water | 20 | 800 | 2.5-3.0 | 44 | 33 | 31 |
| Well Water 1 | 20 | 150 | 5.5 – 6.0 | 5 | 40 | 44 |
| Well Water 1 | 20 | 0 | 7 | 5 | 39 | 38 |
| Well Water 2 | 20 | 700 | 2.5-3.0 | 2 | 10 | 16 |
| Well Water 2 | 20 | 800 | 2.5-3.0 | 2 | 12 | 19 |
| CCWWTP outfall | 30 | 200 | 5.0 – 5.5 | 3 | 1 | 6 |
| CCWWTP outfall filtered | 20 | 400 | 4.5 | 8 | 2 | 16 |

# SPE observations

- Drying time matters – 20 min best
- pH matters  - pH 5 to 5.5 best
- DI water fairly good recoveries
- “Real” waters not so good recoveries especially for azithromycin. Why? Possible interferences with SPE resins
  - Surfactants
  - Iron oxide
  - Polymers?

# Extraction Recoveries PLE

ASE spikes 250ng, 0.5 g sample

| | % avg recoveries (%rsd, n) | | |
|---|---|---|---|
| | Azithromycin | Roxithromycin | Clarithromycin |
| Milorganite | 28 (3%, 4) | 19 (25%, 4) | 54 (2%, 4) |
| LA Biosolids | 16 (-, 2) | 1 (-, 2) | 13 (-, 2) |
| CCWWTP sediments | 3.5 (86%, 4) | 73 (31%, 4) | 106 (25%, 4) |
| CCWWTP plant | 29 (-, 2) | 17 (-, 2) | 17 (-, 2) |
| CCWWTP root | 43 (-, 2) | 30 (-, 2) | 16 (-, 2) |

# Water

| Site(s) | Azithromycin Range in ng/L | Clarithromycin Range in ng/L | Roxithromycin Range in ng/L |
|---|---|---|---|
| Maine* | ND – 77 | NA | NA |
| Connecticut* | ND - 39 | NA | NA |
| Clark County WWTP | | | |
| POCIS† | | | |
| June/July 2002†† | 15 | NA | ND |
| Jan/Feb 2003†† | 66 | | |
| SPE (Aug 2006)†† | 6 | 7.3 | ND |

NA = not analyzed; ND = not detected; †† Methamphetamine detected: 1 ng/L, 1ng/L, and 2 ng/L, respectively. MDMA detected SPE Aug 2006 at 6 ng/L.

* Complete data found in Jones-Lepp TL. 2006. "Chemical markers of human waste contamination: Analysis of urobilin and pharmaceuticals in source waters," J Environ Monit. 8, 472–478 ; † Complete data found in Jones-Lepp TL, Alvarez D, Petty J, Huggins J. 2004. "Polar Organic Chemical Integrative Sampling (POCIS) and LC-ES/ITMS for Assessing Selected Prescription and Illicit Drugs in Treated Sewage Effluents," Archives Environ Cont Toxicol, 47(4), 427-439

# Biosolids

| Site(s) | Azithromycin ng/g dry wt | Clarithromycin ng/g dry wt | Roxithromycin ng/g dry wt |
|---|---|---|---|
| City of Milwaukee (Milorganite®) | 14 (51)* | 9 (18) | 0.4 (2) |
| City of Los Angeles Hyperion WWTP** | 25 (152) | 20 (160) | nd |
| City of Las Vegas WWTP† | 16 | nd | nd |

NA = not analyzed; ND = not detected ; *value in (-) reflects a “corrected” value based on % recoveries from each biosolids material. **Methamphetamine detected at 4 ng/g; †Methamphetamine detected at 5 ng/g.

# Sediments & Plants

| Clark County WWTP outfall – August 2006 | Azithromycin ng/g dry wt | Roxithromycin ng/g dry wt | Clarithromycin ng/g dry wt |
|---|---|---|---|
| Sediment 1 | 1 (28)* | detected < LOQ | 77 |
| Sediment 2 | 2 (64)* | 1 | 97 |
| Plant material (tentative ID - Nicotiana tabacum; Cultivated Tobacco) | 2 | nd | nd |
| Root material | 39 | nd | nd |

nd = not detected; *value in (-) reflects a “corrected” value base on % recoveries from each solid material

# Conclusions

- Preliminary data suggests that there are reservoirs of the macrolides other than wastewater and biosolids, i.e., wetland plant/roots and sediments.
  - More wetland plant samples and sediments are needed to strengthen hypothesis.
- Need to improve extraction recoveries from wastewater. Possible interferences from surfactants and wastewater additions (e.g., iron oxide, chlorine, polymers)
- Some correlation between prescribed use of macrolides and environmental findings, but presence of Roxithromycin, which is not used in the U.S., suggests other means are used for obtaining antibiotics.
  - Finding illicit drugs [i.e., methamphetamine, MDMA (Ecstasy)] shows drug use other than just prescription/over-the-counter.

# Acknowledgments

Thanks to Mr. Tom Moy, Senior Environmental Employee (SEE), for all of his hard work and dedication in the laboratory to make this project a success.

# NOTICE

*Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.*